Mechanism of Manganese Peroxidase Compound II Reduction. Effect of Organic Acid Chelators and pH

Katsuyuki Kishi,[‡] Hiroyuki Wariishi,[‡,§] Leah Marquez,[∥] H. Brian Dunford,[∥] and Michael H. Gold*[,‡]

Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science & Technology, Portland, Oregon 97291, and Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

Received March 22, 1994; Revised Manuscript Received May 19, 1994[⊗]

ABSTRACT: The effect of oxalate, malonate, lactate, and succinate chelators on the reduction of *Phanerochaete chrysosporium* manganese peroxidase compound II by Mn^{II} was investigated using stopped-flow techniques. All rate data were collected from single-turnover experiments under pseudo-first-order conditions. With oxalate, the reduction of compound II by Mn^{II} exhibited saturation behavior when the observed pseudo-first-order rate constants were plotted against oxalate concentration. The plots passed through the origin, indicating that the reduction by Mn^{II} is irreversible at all concentrations of oxalate. Maximal stimulation of the rate of compound II reduction occurred at 2 mM oxalate, the concentration of oxalate found in the extracellular medium of agitated cultures of this fungus. In contrast, maximal stimulation of the reduction of compound II by Mn^{II} only was observed at high (>20 mM) nonphysiological concentrations of malonate and lactate. Furthermore, at low concentrations of malonate and lactate, the reduction of compound II appeared to be reversible. These results suggest that at physiological concentrations oxalate chelates and stabilizes Mn^{III}, enhancing its efficient removal from the enzyme. The rate constants for compound II reduction exhibited bell-shaped curves as a function of pH and had optima at pHs 5.0–5.4. In the presence of succinate, triphasic kinetics were observed for compound II reduction by Mn^{II}. In contrast to the reduction of compound II by Mn^{II}, various chelators had no observable effect on the formation of compound I. However, they did affect the steady-state oxidation of 2,6-dimethoxyphenol.

White rot basidiomycete fungi are primarily responsible for the initiation of the decomposition of lignin in wood (Gold et al., 1989; Kirk & Farrell, 1987; Buswell & Odier, 1987). When cultured under ligninolytic conditions, the white-rot fungus *Phanerochaete chrysosporium* produces two extracellular heme peroxidases, LiP and MnP,[1] which along with an H_2O_2-generating system are major components of its lignin degradative system (Kirk & Farrell, 1987; Buswell & Odier, 1987; Tien, 1987; Hammel & Moen, 1991; Wariishi et al., 1991b; Kuwahara et al., 1984). MnP has been purified and characterized. The enzyme contains one iron protoporphyrin IX prosthetic group, is a glycoprotein of M_r ~46 000, and exists as a series of isoenzymes (Glenn & Gold, 1985; Paszczynski et al., 1986; Leisola et al., 1987; Mino et al., 1988; Wariishi et al., 1988; Harris et al., 1991). Spectroscopic studies and cDNA sequences reveal that the heme environment of manganese peroxidase is similar to that of other plant and fungal peroxidases (Glenn & Gold, 1985; Mino et al., 1988; Harris et al., 1991; Dunford & Stillman, 1976; Banci et al., 1992; Pribnow et al., 1989; Pease et al., 1989). In addition, kinetic and spectral characterization of the oxidized intermediates, compounds I and II, indicates that the catalytic cycle of MnP is similar to that of horseradish peroxidase and LiP (Gold et al., 1989; Wariishi et al., 1988, 1989, 1992; Renganathan & Gold, 1986; Glenn et al., 1986). Importantly, the enzyme oxidizes Mn^{II} to Mn^{III}, and the latter, in turn, oxidizes phenolic substrates, including lignin model compounds (Tuor et al., 1992), lignin (Wariishi et al., 1991b), and chlorinated phenols (Joshi & Gold, 1993). Transient state kinetic analysis has confirmed that Mn^{II}/Mn^{III} acts as a redox couple rather than as an enzyme binding activator (Wariishi et al., 1989). Thus, enzymatically generated Mn^{III} is utilized as a freely diffusible oxidant, enabling the enzyme to oxidize polymeric lignin within the woody matrix (Wariishi et al., 1988, 1992; Glenn et al., 1986; Tuor et al., 1992; Joshi & Gold, 1993).

We previously have emphasized the importance of organic acid chelators in the manganese peroxidase system. Organic acids, including lactate, malonate, and oxalate, chelate enzymatically generated Mn^{III}, stabilizing this species in aqueous solution (Glenn & Gold, 1985; Paszczynski et al., 1986; Wariishi et al., 1989; Demmer et al., 1980), and, as we shall show, ensuring the efficiency of Mn^{II} oxidation. Recently, the production and secretion of oxalate and malonate by *P. chrysosporium* were demonstrated (Wariishi et al., 1992; Barr et al., 1992; Kuan & Tien, 1993; Dutton et al., 1992). In particular, the production of millimolar concentrations of oxalate by this fungus under defined conditions was reported (Wariishi et al., 1992; Barr et al., 1992; Kuan & Tien, 1993; Dutton et al., 1992). We previously reported that relatively high concentrations of malonate or lactate are required to stimulate optimal MnP activity (Glenn & Gold, 1985; Paszczynski et al., 1986; Wariishi et al., 1992); however, these high concentrations are not physiological (Wariishi et al., 1992; Kuan & Tien, 1993). Recently it was shown that optimal enzyme activity is obtained in the presence of 500 μM oxalate, a concentration found in *P. chrysosporium* cultures (Kuan & Tien, 1993); a kinetic analysis was published in a subsequent paper (Kuan et al., 1993). In the present study, we reinves-

* To whom correspondence should be addressed at the Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science & Technology, P.O. Box 91000, Portland, OR 97291-1000. Telephone: 503-690-1076. Fax: 503-690-1464.

‡ Oregon Graduate Institute of Science & Technology.

§ Present address: Department of Forest Products, Kyushu University, Fukuoka 812, Japan.

∥ University of Alberta.

⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1994.

[1] Abbreviations: MnP, manganese peroxidase; LiP, lignin peroxidase; *m*CPBA, *m*-chloroperoxybenzoic acid; 2,6-DMP, 2,6-dimethoxyphenol; HPLC, high-performance liquid chromatography.

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tigated the effect of several organic acid chelators under a variety of conditions which shed light upon the behavior of both Mn^{II} and Mn^{III} in the MnP system. Our results confirm that physiological concentrations of oxalate stimulate the reduction of the oxidized enzyme intermediates and probably stabilize the enzyme-generated Mn^{III} by chelation. In addition, we report detailed analysis of the pH dependence of the overall MnP reaction. We show that succinate exhibits a complex behavior, in contrast to a report that it has little effect on the reactions of MnP (Kuan et al., 1993).

MATERIALS AND METHODS

Chemicals. H_2O_2 (30% solution) was obtained from BDH Chemicals. *m*CPBA, 2,6-DMP, and veratryl alcohol were obtained from Aldrich. The concentrations of H_2O_2 and *m*CPBA stock solutions were determined as described (Cotton & Dunford, 1973). All other chemicals were reagent grade. Solutions were prepared using deionized water obtained from a Milli Q system (Millipore).

Enzyme Preparation. MnP isozyme 1 was purified from the extracellular medium of acetate-buffered agitated cultures of *P. chrysosporium* strain OGC101 (Alic et al., 1987) as described (Glenn & Gold, 1985; Wariishi et al., 1989). The purified enzyme was electrophoretically homogeneous and had an RZ (A_{406}/A_{280}) value of 6. The enzyme concentration was determined at 406 nm using an extinction coefficient of 129 mM^{-1} cm^{-1} (Glenn & Gold, 1985). Oxalate oxidase and horseradish peroxidase were purchased from Sigma. LiP was assayed utilizing veratryl alcohol as described (Gold et al., 1989; Kirk & Farrell, 1987). MnP was routinely assayed by following the formation of Mn^{III}–malonate as described (Glenn & Gold, 1985; Glenn et al., 1986).

Oxalate Produced by P. chrysosporium. *P. chrysosporium* was grown from a conidial inoculum at 38 °C in shaking cultures, with 2% glucose and 1.2 mM ammonium tartrate as the carbon and nitrogen sources, as described (Wariishi et al., 1992; Kirk et al., 1978). The extracellular concentration of oxalate produced in the cultures was determined via two independent methods: (i) using HPLC (Wariishi et al., 1992) and (ii) using oxalate oxidase as previously described (Barr et al., 1992; Kuan & Tien, 1993; Laker et al., 1980) except that 2,6-DMP was used as the peroxidase substrate.

Kinetic and Rapid-Scan Spectral Measurements. Kinetic measurements were conducted at 25.0 ± 0.5 °C using a Photal RA601 Rapid Reaction Analyzer. One reservoir contained the enzyme in water at a concentration of 2 μM for MnP compound I formation and 5 μM for compound II reduction experiments. The other reservoir contained the substrate (H_2O_2 or Mn^{II}), in buffer, in at least 10-fold excess. Experiments were performed with the following buffers as indicated: potassium malonate, potassium oxalate, potassium lactate, and potassium succinate (ionic strength 0.1 adjusted with K_2SO_4). The pH was varied from 3.34 to 6.95. The final pH of reactions was measured using an Accumet 50 pH meter (Fisher Scientific). Compound II was formed by the successive addition of 1.0 equiv of *m*CPBA and 0.9 equiv of potassium ferrocyanide to the native enzyme. Compound II samples were freshly prepared for each experiment. All of the kinetic traces exhibited single-exponential character from which pseudo-first-order rate constants were calculated. In each kinetic experiment, several substrate concentrations were used, and plots of pseudo-first-order rate constants versus substrate concentrations were obtained.

Rapid-scan spectra were recorded on a multichannel photodiode array (Photal RA601) equipped with a 1-cm observation cell at 25.0 ± 0.5 °C. The spectral region from 345 to 445 nm was scanned. Electronic absorption spectra obtained on a conventional time scale were recorded on a Shimadzu UV-260 or Beckman DU 650 spectrophotometer.

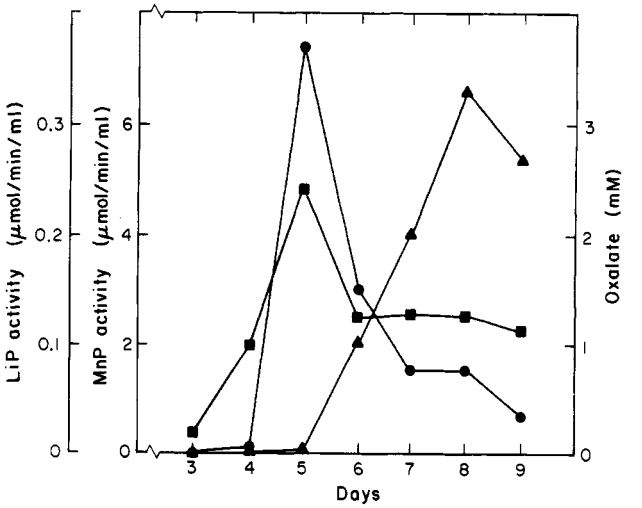

FIGURE 1: Production of manganese and lignin peroxidases and oxalate in agitated cultures of *P. chrysosporium*. Oxalate concentrations (■) and lignin (▲) and manganese peroxidase activities (●) in the extracellular medium were assayed as described in the text.

Oxidation of 2,6-Dimethoxyphenol by MnP. Oxidation of 2,6-DMP by MnP was followed spectrophotometrically at 469 nm for quinone dimer formation (Wariishi et al., 1992). Reaction mixtures (1 mL) contained manganese peroxidase (0.5 μg), $MnSO_4$ (0.5 mM), 2,6-DMP (0.2 mM), and H_2O_2 (0.1 mM) in each of the buffers described above (ionic strength 0.1).

RESULTS

Enzyme Preparation. The effect of freeze–thaw cycles on manganese peroxidase activity was examined. One freeze–thaw cycle of the enzyme (1 mg/mL in water) resulted in an activity loss of ~12%, and five cycles resulted in an activity loss of 50%. The freeze–thaw-treated enzyme showed a similar Soret and visible absorption spectrum to the untreated enzyme. In our previous transient state kinetic study (Wariishi et al., 1989), MnP was stored frozen, and the stock solution was thawed whenever enzyme was needed. Probably owing to this treatment, smaller rate constants were reported for compound I formation and compound II reduction (Wariishi et al., 1989) as compared to those found in the present study. However, the previously reported kinetic features such as saturation phenomenon and reversibility were similar to those described below. In our present work, we utilized freshly prepared enzyme and eliminated freeze–thaw cycles.

Fungal Secretion of Oxalate and Peroxidases. A time course for oxalate and peroxidase accumulation in the extracellular medium of shaking cultures of *P. chrysosporium* is shown in Figure 1. Under these conditions, oxalate accumulation reached a maximum of 2.4 mM on day 5 and decreased on day 6, leveling off at ~1 mM. Extracellular MnP activity also was maximal on day 5 and decreased thereafter. In contrast, LiP activity first appeared on day 6, when the oxalate concentration was decreasing, and was maximal on day 8 (Figure 1).

Effect of Chelators on the Oxidation of 2,6-DMP by MnP. The rate dependence of 2,6-DMP oxidation by Mn^{III}, generated by the MnP system, on the concentrations of malonate and oxalate is shown in Figure 2. Enzyme activity increased with

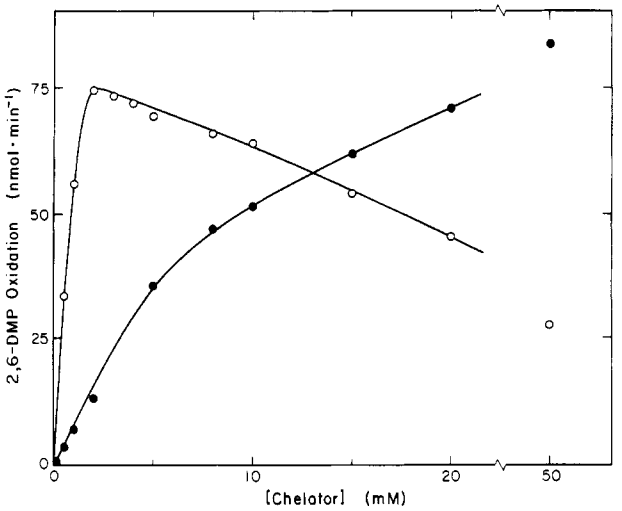

FIGURE 2: Effect of the chelator concentration on 2,6-dimethoxyphenol oxidation by MnP. Reaction mixtures contained MnP (0.5 μg/mL), $MnSO_4$ (0.5 mM), 2,6-DMP (0.2 mM), and H_2O_2 (0.1 mM) in oxalate (○) and malonate (●) at pH 4.5. The reaction was followed by measuring the increase in absorbance at 469 nm, indicating quinone dimer formation (Wariishi et al., 1992).

increasing malonate concentration, reaching a maximum at ~50 mM. Similar results were observed with lactate although the maximal activity in 50 mM lactate was ~80% of that in 50 mM malonate (data not shown). In contrast, maximum enzyme activity was observed with 2 mM oxalate. At concentrations above 2 mM, enzyme activity slowly decreased with increasing oxalate concentrations. The maximum MnP activity in 2 mM oxalate was ~90% of that in 50 mM malonate. No 2,6-DMP oxidation by manganese peroxidase was observed in reactions conducted in succinate buffer (Wariishi et al., 1992). These results are consistent with our previous studies conducted with chelator concentrations of 50 mM (Wariishi et al., 1992).

Reduction of Compound II in the Presence of Oxalate. When the reduction of compound II was examined at low concentrations of potassium oxalate (1–5 mM), the plot of the observed rate constants versus [Mn^{II}] leveled off at high [Mn^{II}]. This reaction can be described by a simple binding interaction between the reactants according to eqs 1–3:

$$\text{compound II} + \text{Mn}^{\text{II}}\text{-complex} \overset{K_2}{\rightleftarrows} \text{compound II–Mn}^{\text{II}}\text{-complex} \overset{k_2}{\rightarrow} \text{native enzyme} + \text{Mn}^{\text{III}}\text{-complex} \quad (1)$$

$$k_{2obs} = \frac{k_2}{1 + K_2/[\text{Mn}^{\text{II}}\text{-complex}]} \quad (2)$$

$$K_2 = \frac{[\text{compound II}][\text{Mn}^{\text{II}}\text{-complex}]}{[\text{compound II–Mn}^{\text{II}}\text{-complex}]} \quad (3)$$

where k_2 is a first-order rate constant (s^{-1}) and K_2 is a dissociation constant (M). The calculated values for k_2 and K_2 are listed in Table 1.

When the reaction was conducted in higher concentrations of oxalate (10 and 20 mM), the plot of observed rate constants versus Mn^{II} concentration was linear and passed through the origin within experimental error (Figure 3), indicating that, under these conditions, the reduction of compound II obeyed second-order kinetics and was irreversible. The second-order rate constants were calculated from the slopes of the plots in Figure 3 and are listed in Table 1. Saturation behavior might be expected at higher concentrations of Mn^{II}.

Reduction of Compound II in Malonate and Lactate Buffers. The reduction of MnP compound II to native enzyme was followed at 406 nm in potassium malonate, pH 4.60, and in potassium lactate, pH 4.56, under pseudo-first-order conditions with the reducing substrate, Mn^{II}, in excess. When the reaction was conducted in either 10 or 20 mM potassium malonate, the plot of the observed rate constants (k_{2obs}) versus Mn^{II} concentration leveled off at high Mn^{II} concentration (Figure 4A), suggesting a binding interaction between reactants. In contrast, when the reaction was conducted in lower concentrations of malonate (1–5 mM), the dependence of k_{2obs} on substrate concentration (0–100 μM) was linear, and the plot had a positive y-axis intercept (Figure 4B), suggesting that, at these concentrations, the reaction was reversible as expressed by eqs 4 and 5:

$$\text{compound II} + \text{Mn}^{\text{II}}\text{-complex} \underset{k_{-2}}{\overset{k_{2app}}{\rightleftarrows}} \text{native enzyme} + \text{Mn}^{\text{III}}\text{-complex} \quad (4)$$

$$k_{2obs} = k_{2app}[\text{Mn}^{\text{II}}\text{-complex}] + k_{-2} \quad (5)$$

where k_{2app} is a second-order rate constant for the forward reaction and k_{-2} is a first-order rate constant for the reverse reaction. The kinetic parameters are summarized in Table 1. Similar behavior was observed in potassium lactate, where saturation kinetics were observed at a 20 mM chelator concentration and a reversible reaction was observed at 2 mM (data not shown). All data are summarized in Table 1. Saturation kinetics for this reaction in 20 mM lactate have been reported previously (Wariishi et al., 1989).

Reduction of Compound II in Succinate Buffer. The reduction of compound II by Mn^{II} also was carried out in 20 mM potassium succinate (pH 4.58), which does not chelate Mn^{III} readily (Wariishi et al., 1989, 1992; Demmer et al., 1980). The resultant kinetic traces exhibited a triphasic character (Figure 5). The plots of k_{obs} versus Mn^{II} concentration for each reaction phase are shown in Figure 6. The first phase of the reaction (phase I) displayed saturation kinetics (Figures 5 and 6). In the second phase (phase II), k_{obs} was linearly dependent on Mn^{II} concentration, and the plot had a positive y-intercept (Figure 6), suggesting that the reduction of compound II in phase II was reversible. Approximately 60% of compound II was converted to native enzyme in phases I and II. In the third phase of the reaction (phase III), k_{obs} values were independent of the Mn^{II} concentration in the range 20–100 μM (Figure 6). The kinetic parameters listed in Table 1 show that the rates for the reactions carried out in succinate buffer are slower than the rates in other buffers. The rate for the phase III reaction in succinate is particularly slow.

The rapid-scan spectrum for the conversion of compound II to native enzyme in potassium succinate displayed an apparent isosbestic point at 417 nm (data not shown). This isosbestic point also was observed in the conversion of compound II to native MnP by Mn^{II} in malonate, lactate, and oxalate (data not shown). Thus, the triphasic plot for reactions carried out in succinate apparently was not caused by the contamination of compound II with compound I or with unreacted *m*CPBA. Our results with succinate are in contrast to an earlier report that succinate has little effect on the reaction (Kuan et al., 1993).

pH Dependence of Compound II Reduction. The pH dependence of the reduction of compound II by Mn^{II} also was

Table 1: Kinetic Parameters for the Reduction of MnP Compound II by Mn^{II} in Several Different Organic Acid Chelators[a]

		hyperbolic[b]		linear[c]	
organic acid (pH)	concn (mM)	first-order rate constant (s^{-1})	equilibrium dissociation constant (M)	forward second-order rate constant (M^{-1} s^{-1})	reverse first-order rate constant (s^{-1})
oxalate (4.62)	1	$(2.3 \pm 0.2) \times 10^2$	$(5.6 \pm 1.0) \times 10^{-5}$		
	2	$(2.9 \pm 0.1) \times 10^2$	$(8.7 \pm 0.7) \times 10^{-5}$		
	5	$(5.6 \pm 0.6) \times 10^2$	$(4.4 \pm 0.6) \times 10^{-4}$		
	10			$(6.2 \pm 0.2) \times 10^5$	
	20			$(3.3 \pm 0.1) \times 10^5$	
malonate (4.60)	1			$(1.8 \pm 0.1) \times 10^5$	$(1.8 \pm 0.1) \times 10$
	2			$(6.0 \pm 0.1) \times 10^5$	$(2.1 \pm 0.1) \times 10$
	5			$(9.1 \pm 0.4) \times 10^5$	$(2.1 \pm 0.2) \times 10$
	10	$(3.6 \pm 0.3) \times 10^2$	$(1.9 \pm 0.2) \times 10^{-4}$		
	20	$(8.9 \pm 0.5) \times 10^2$	$(1.9 \pm 0.1) \times 10^{-4}$		
lactate (4.56)	2			$(3.9 \pm 0.2) \times 10^5$	$(2.3 \pm 0.1) \times 10$
	20	$(1.2 \pm 0.3) \times 10^3$	$(9.0 \pm 0.3) \times 10^{-4}$		
succinate I[d] (4.58)	20	$(5.5 \pm 0.6) \times 10$	$(2.4 \pm 0.9) \times 10^{-5}$		
succinate II				$(6.7 \pm 0.7) \times 10^4$	6.9 ± 0.4
succinate III		$(2.3 \pm 0.3) \times 10^{-1}$ [e]			

[a] Data obtained by fits to the plots of the pseudo-first-order rate constant, k_{obs}, vs $[Mn^{II}]$. [b] A nonlinear least-squares fit was applied to the data showing saturation kinetics. [c] A linear least-squares fit was applied to the data showing a linear relationship. [d] The reduction of compound II by Mn^{II} in succinate occurred via triphasic kinetics (Figure 5). [e] The first-order rate for the phase III reaction was $[Mn^{II}]$ independent (Figure 6C).

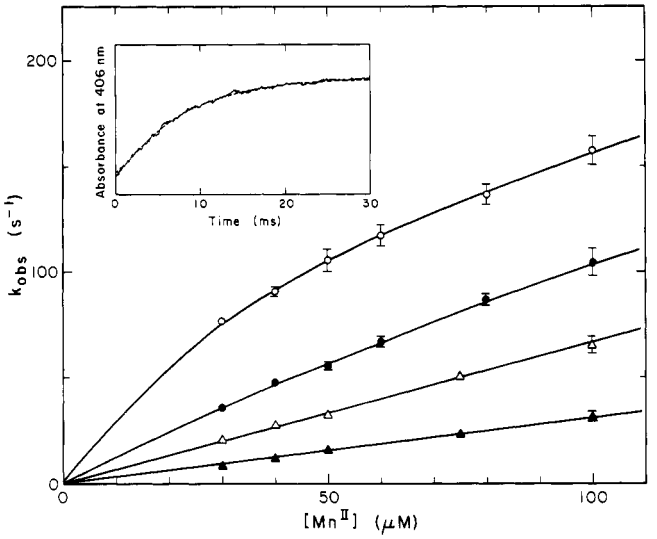

FIGURE 3: Reduction of manganese peroxidase compound II by Mn^{II} in oxalate at pH 4.62. Observed pseudo-first-order rate constants were plotted against $[Mn^{II}]$ at 2 mM (○), 5 mM (●), 10 mM (△), and 20 mM (▲) oxalate. *Inset*: Typical kinetic trace for the reduction of compound II by Mn^{II} (50 μM) in 2 mM oxalate, showing single-exponential character from which a pseudo-first-order rate constant was calculated. 5 μM MnP converted to compound II as described in the text, ionic strength 0.1 M.

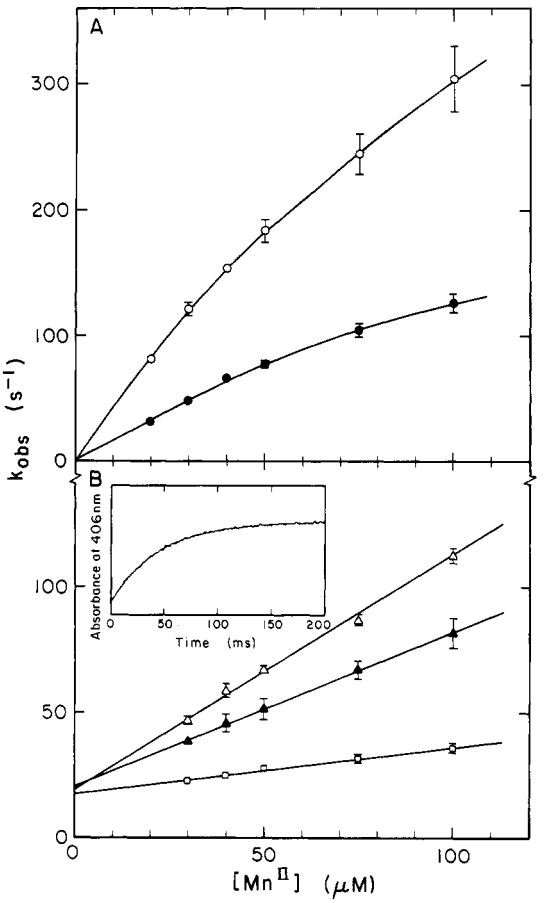

FIGURE 4: Reduction of MnP compound II by Mn^{II} in malonate at pH 4.60. Observed pseudo-first-order rate constants were plotted against $[Mn^{II}]$ at 10 mM (●) and 20 mM (○) malonate (A); and at 1 mM (□), 2 mM (▲), and 5 mM (△) malonate (B). Reaction conditions were as described in the text. *Inset*: Typical trace at 406 nm of compound II reduction by 75 μM Mn^{II} in 5 mM malonate. The curve exhibited single-exponential character. Concentration of enzyme, 5 μM; ionic strength, 0.1.

examined. At low Mn^{II} concentrations, k_{2obs} was linearly proportional to Mn^{II} concentration in reactions carried out in 2 mM oxalate and in 20 mM malonate (Figures 3 and 4). Therefore, the k_{app} at low Mn^{II} concentrations (20–30 μM) was measured over the pH range of 3.34–6.95. For these experiments k_{2obs} was measured using six different Mn^{II} concentrations in 20 mM malonate at pH 3.53, 4.60, 4.99, and 5.99. All reactions displayed saturation kinetics with no apparent reverse reaction (data not shown). The pH dependence of k_{app} ($k_{2obs}/[Mn^{II}]$) for compound II reduction is shown in Figure 7. Maximum rates were obtained at pHs 5.0 and 5.4 in 2 mM oxalate and 20 mM malonate, respectively. The pH profiles for compound II reduction by Mn^{II} (Figure 7) were similar to those for oxidation of 2,6-DMP (data not shown).

pH Dependence of 2,6-DMP Oxidation. The pH profiles for 2,6-DMP oxidation by Mn^{III}, produced by the action of MnP, exhibited maxima at pH 4.56 in 2 mM oxalate and at pH 4.60 in 20 mM malonate (data not shown). Above the optimal pH the decrease in MnP activity was steeper in oxalate than in malonate. In contrast, below the optimal pH, the decrease in MnP activity was steeper in malonate than in oxalate. These differences suggest that organic acid chelators may be controlling the pH dependence of the reactions.

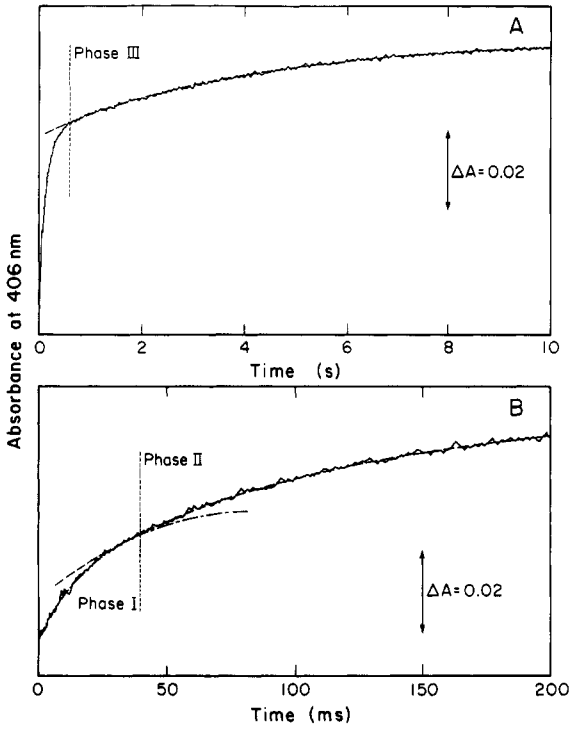

FIGURE 5: Time course of the reduction of MnP compound II by Mn^{II} in succinate. (A) Kinetic trace for the reduction of compound II by Mn^{II} (50 μM) in 20 mM succinate, pH 4.58. The sampling period was 10 s. (B) The same as (A) except a sampling period of 200 ms was used. Dashed lines are computer-fitted single-exponential curves from which observed pseudo-first-order rate constants were calculated for each phase of the triphasic reaction.

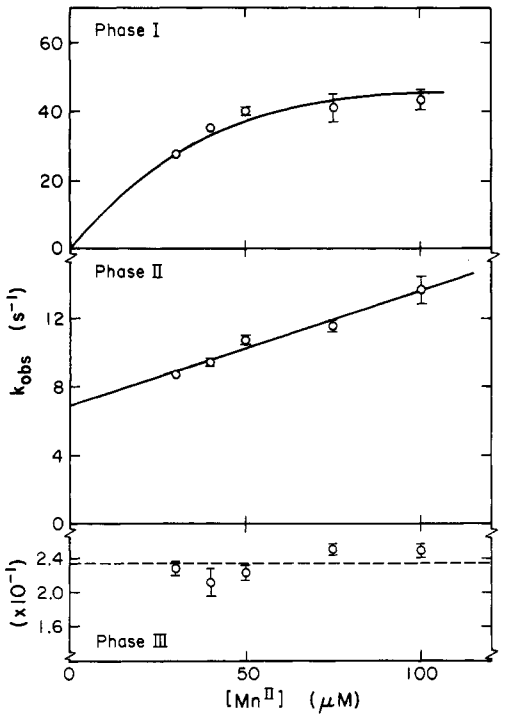

FIGURE 6: Reduction of MnP compound II by Mn^{II} in succinate at pH 4.58. Analysis of each phase of the triphasic reaction. Observed pseudo-first-order rate constants k_{obs} are plotted against [Mn^{II}] for each of phases I, II, and III.

Rate of Compound I Formation. The rate of MnP compound I formation from native enzyme and H_2O_2 was determined at pH 4.6 in oxalate, malonate, lactate, and succinate buffers. Compound I formation was measured at 397 nm, the isosbestic point between compounds I and II, excluding interference from the possible conversion of compound I to II. Each trace displayed single-exponential character. The observed rate constants (k_{1obs}) were linearly proportional to the H_2O_2 concentrations at 10–50-fold excess. The second-order rate constants (k_{1app}) for compound I formation (Table 2) were not affected by the type or concentration of the organic acid.

DISCUSSION

We have confirmed (Wariishi et al., 1989) that the reduction rate for MnP compound I by Mn^{II} is >20 times faster than that for compound II (data not shown). Therefore, provided sufficient hydroperoxide is present, the rate-determining step in the catalytic cycle is the reduction of compound II to native enzyme. Using only 1.0 equiv of *m*CPBA and 0.9 equiv of ferrocyanide to generate compound II assures a single turnover. Thus, the rate of compound II reduction can be measured directly at 406 nm, the Soret maximum for native MnP.

Effect of Chelators on MnP Compound II Reduction. (A) Oxalate. In oxalate, the pseudo-first-order rate constant k_{obs} for compound II reduction decreases as the concentration of the chelator increases beyond 2 mM (Figure 3), which is consistent with the effect of oxalate concentration on the MnP oxidation of 2,6-DMP (Figure 2). A plausible explanation is that the dioxalate complex of Mn^{II} binds less well to the enzyme than the monooxalate complex. At low concentrations (1, 2, and 5 mM) of oxalate, plots of k_{obs} versus Mn^{II} concentration are hyperbolic, requiring the introduction of a binding constant to fit the data quantitatively (eqs 1–3). In contrast, at higher concentrations of oxalate, the reaction does not show saturation phenomena (Figure 3). If the dioxalate complex of Mn^{II} is oxidized, then Mn^{III} may be released faster since further chelation is apparently not required. However, at sufficiently high concentrations of Mn^{II}, one would expect the linear plots of k_{obs} versus Mn^{II} concentration at 20 mM oxalate to exhibit curvature, showing the same behavior observed at lower oxalate concentrations. Thus, the data displayed in Figure 3 are consistent with a smooth transition from lowest to highest chelator concentration. In contrast to the results obtained with malonate and lactate, the reduction of compound II by Mn^{II}-oxalate shows saturation kinetics at low concentrations of oxalate (Figure 3). Furthermore, at either low or high concentrations of oxalate, the reduction of compound II is irreversible.

(B) Malonate and Lactate. The value of k_{obs} for compound II reduction increases with increasing concentration of malonate (Figure 4), consistent with the effect of malonate on the oxidation of 2,6-DMP (Figure 2). At high concentrations of malonate (10 and 20 mM), the plots of k_{obs} versus Mn^{II} concentration are hyperbolic (Figure 4A). Nonlinear saturation responses have been observed previously for the reactions of MnP compounds I and II with Mn^{II} in lactate (Wariishi et al., 1989), for the reductions of horseradish peroxidase compounds I and II with *p*-cresol (Hewson & Dunford, 1976; Critchlow & Dunford, 1972) and *p*-aminobenzoic acid (Dunford & Cotton, 1975), and for the reduction of LiP compound II with veratryl alcohol (Wariishi et al., 1991a). This can be attributed to the binding interaction between the enzyme and substrate followed by enzyme reduction (eqs 1–3). In contrast, when the reaction is conducted in low concentrations of malonate (Figure 4B), plots of k_{obs} versus [Mn^{II}] are linear. These differences imply different lifetimes for the compound II–Mn^{II} complex dependent upon the concentration of malonate. Furthermore, at low concentrations of malonate, the reaction is reversible,

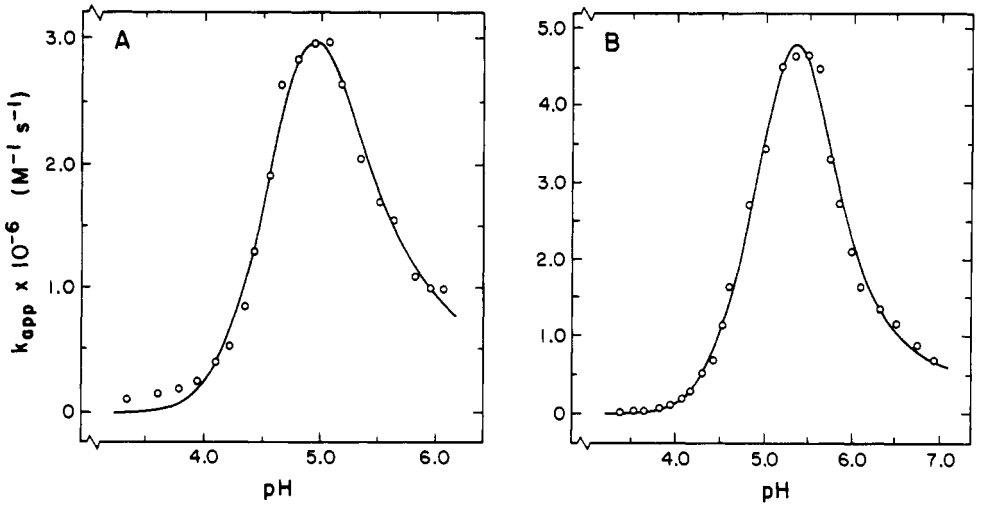

FIGURE 7: pH dependence of the reduction of MnP compound II by Mn^{II} in oxalate and malonate. Observed pseudo-first-order rate constants divided by $[Mn^{II}]$ generated second-order rate constants which are plotted as a function of pH. The solid curves are calculated on the basis of two rate constants, two ionization constants on the enzyme, and one ionization constant of the chelator. All parameters have the same value for both curves within experimental error, except the constants calculated for the chelators: pK values of 4.5 and 5.6 for oxalate and malonate, which agree within experimental error with their pK_{a2} values. (A) Reduction of MnP compound II in 2 mM oxalate, pH 4.62; (B) reduction of MnP compound II in 20 mM malonate, pH 4.60.

Table 2: Rate of Formation of Manganese Peroxidase Compound I in the Presence of Several Different Chelators

organic acid (pH)	concn (mM)	k_{app}[a] (M^{-1} s^{-1})
oxalate (4.62)	2	$(3.9 \pm 0.1) \times 10^6$
	20	$(3.8 \pm 0.1) \times 10^6$
malonate (4.60)	2	$(3.6 \pm 0.2) \times 10^6$
	20	$(3.6 \pm 0.1) \times 10^6$
lactate (4.56)	2	$(3.6 \pm 0.5) \times 10^6$
	20	$(3.6 \pm 0.3) \times 10^6$
succinate (4.58)	2	$(3.4 \pm 0.4) \times 10^6$
	20	$(3.8 \pm 0.1) \times 10^6$

[a] MnP compound I formation was followed at 397 nm, the isosbestic point between compounds I and II. Traces showed single-exponential character from which pseudo-first-order rate constants were calculated. These rate constants were linearly proportional to $[H_2O_2]$ with zero intercept.

and the rate of the reverse reaction is essentially independent of malonate concentration (1, 2, and 5 mM). These differences in the kinetic features of the reaction at high and low chelator concentrations also are observed for compound II reduction in lactate (Table 1). The reversibility of the reaction in low malonate and lactate concentrations may be explained as follows. At low concentrations of malonate and lactate, the Mn^{III} produced by compound II may not be chelated effectively and thus is not stabilized. Owing to the high redox potential of the Mn^{II}/Mn^{III} couple in water (1.54 V), reoxidation of native enzyme to compound II by Mn^{III} may occur. The single-step, one-electron oxidation of native horseradish peroxidase to compound II by the $[IrCl_6]^{3-}/[IrCl_6]^{2-}$ couple, which has a reduction potential of 0.93 V, has been reported (Hayashi & Yamazaki, 1979).

(C) Succinate. The rate of reduction of compound II in succinate is triphasic. Three successive exponential curves are observed when the absorbance change is plotted versus time, with the rate becoming progressively slower (Figure 5). As shown in Figure 6, the phase I reaction exhibits saturation kinetics with excess Mn^{II}. In phase II, the plot of k_{obs} versus Mn^{II} is linear with a finite intercept. In phase III of the reaction in succinate, the values of k_{obs} are extremely low ($\sim 2.4 \times 10^{-1}$ s^{-1}) and independent of Mn^{II} concentrations in the range from 20 to 100 μM (Figure 6). The reverse reaction, the oxidation of native enzyme to compound II by Mn^{III}, seems to occur with weak chelators such as succinate (Wariishi et al., 1989; Demmer et al., 1980) which apparently are unable to form a stable complex with Mn^{III}. The lack of 2,6-DMP oxidation by MnP in succinate (Wariishi et al., 1992) can be explained by this ineffective chelation and hence stabilization of Mn^{III} (Wariishi et al., 1988).

pH Dependence of Compound II Reduction and 2,6-DMP Oxidation. The pH optimum for oxidation of phenolic substrates by MnP is ~4.5 (Glenn & Gold, 1985; Paszczynski et al., 1986; Glenn et al., 1986; Aitken & Irvine, 1990), despite a higher reactivity of Mn^{III} complexes at lower pHs (Glenn et al., 1986; Demmer et al., 1980), suggesting that the pH dependency is at least partially controlled by enzyme-bound ionizable groups. Compound II reduction is usually rate-controlling. Therefore, in order to elucidate the factors involved, we have examined the pH dependence of compound II reduction in the presence of malonate and oxalate.

For the reduction of compound II by Mn^{II}, the plots of k_{app} versus pH in 2 mM oxalate and 20 mM malonate both exhibit a sharp peak at the pH optimum (Figure 7). The optimum is pH 5.0 in oxalate and pH 5.4 in malonate, both of which are slightly higher than the optimal pH for oxidation of 2,6-DMP in the same buffers (pH 4.56 and 4.6 for oxalate and malonate, respectively). The lower pH optimum for the oxidation of 2,6-DMP may be a result of the low pH optimum for the reaction of Mn^{III}-complexes with aromatic substrates. For example, a pH optimum of 2.5 has been reported for the oxidation of aromatic substrates by Mn^{III}-lactate (Glenn et al., 1986).

Plots of the pH dependence of compound II reduction in both oxalate and malonate display at least two inflection points. When the logarithm of k_{app} is plotted against pH, a linear region with a slope of ~1.6 is obtained in the pH range 4–5 for both oxalate and malonate. If a single kinetically important ionization occurs, a slope of 1.0 would be expected (Hayashi & Yamazaki, 1979); however, the value of $\log(k_{app})$ increases more rapidly with increasing pH. This observation strongly suggests an additional important ionization in the region where rate increases with increasing pH. The inflection point at higher pH where the rate is decreasing implies a role for another acid group. Thus, three kinetically important ionizations may be involved in the pH range shown in Figure 7. A nonlinear

least-squares program was applied to the data in Figure 7, in which three acid dissociation constants (two for the enzyme, K_{E1} and K_{E2}, and one for the chelator) and three rate constants were introduced as adjustable parameters. It turned out that only two rate constants were required; the value for the third was negligible. All parameters except those for the chelator were essentially the same within experimental error for both oxalate and malonate (Figure 7A,B) and are as follows: $pK_{E1} = 6.1 \pm 0.5$, $pK_{E2} = 3.4 \pm 0.5$, $k_1 = (4.6 \pm 1.0) \times 10^5$ M^{-1} s^{-1} and $k_2 = (1.8 \pm 0.6) \times 10^8$ M^{-1} s^{-1}, with the most acid form of the enzyme being most reactive and the most basic form unreactive. The values for the chelators are $pK = 4.5 \pm 0.2$ for oxalate and 5.6 ± 0.5 for malonate; these are the same within experimental error as the pK_{a2} values for these chelators (4.28 for oxalate and 5.69 for malonate). Undoubtedly, ionizations of oxalate and malonate affect their chelating ability. Specific assignment of dissociation constants to acid groups in the enzyme active site would be premature.

Compound I Formation Rate and Effect of Chelators. The primary reaction product of peroxidases with H_2O_2 is the oxidized intermediate compound I. MnP compound I is similar to other peroxidase compounds I in its spectral features and in the activation energy for its formation (Wariishi et al., 1988, 1989; Dunford & Stillman, 1976; Renganathan & Gold, 1986; Marquez et al., 1988; Hewson & Dunford, 1975; Andrawis et al., 1988; Job et al., 1978). We have shown that the second-order rate constant for MnP compound I formation with H_2O_2 as the substrate is independent of pH over the range 3.1–8.3 (Wariishi et al., 1989). In this study, utilizing a better enzyme preparation, we confirm that the compound I formation rate (k_{1app}) is independent of pH (data not shown) and that k_{1app} is twice as large as that which we reported previously (Table 2) (Wariishi et al., 1989). In addition, the value of k_{1app} is independent of both the type and concentration of the organic acid chelator (Table 2), suggesting that both the pH and chelator dependencies of MnP reactions (Figures 2 and 7) (Glenn & Gold, 1985; Wariishi et al., 1988; Glenn et al., 1986) are dictated by effects on the reductions of MnP compounds I and II.

General Discussion. Kinetic and inhibition studies suggest that MnP has a single binding site for Mn^{II} in the vicinity of the heme (Harris et al., 1991; Wariishi et al., 1992) and that Mn^{II} is the substrate for the enzyme (Harris et al., 1991; Wariishi et al., 1989, 1992). The catalytic activity of MnP is stimulated dramatically by C_2 and C_3 dicarboxylic or α-hydroxy acids such as oxalate, malonate, and lactate (Glenn & Gold, 1985; Wariishi et al., 1988, 1992; Glenn et al., 1986; Kuan & Tien, 1993; Kuan et al., 1993). These organic acid chelators (i) stabilize Mn^{III} in aqueous solution (Wariishi et al., 1988, 1989; Glenn et al., 1986) and (ii) chelate Mn^{II}.

Under ligninolytic conditions, *P. chrysosporium* secretes several organic acid metabolites including oxalate, malonate, citrate, and glyoxylate (Wariishi et al., 1992; Barr et al., 1992; Kuan & Tien, 1993; Dutton et al., 1992). Oxalate, a common secondary metabolite of wood-rotting basidiomycetes (Takao, 1965), is the major organic acid secreted by this fungus (Figure 1) (Wariishi et al., 1992; Barr et al., 1992; Kuan & Tien, 1993; Dutton et al., 1992). Furthermore, oxalate is the only organic acid secreted at the concentrations required to stimulate MnP (Kuan & Tien, 1993). Maximal production of oxalate and MnP occurs simultaneously in agitated cultures of *P. chrysosporium* (Figure 1). Furthermore, maximal enzyme activity is observed with 2 mM oxalate, approximately the maximal oxalate concentration secreted by the fungus in agitated culture (Figures 1 and 2). In contrast, maximal MnP activity requires 50 mM malonate (Figure 2) (Wariishi et al., 1992) although the concentration of malonate secreted by this organism in agitated cultures is approximately 20–30 μM (Wariishi et al., 1992). High concentrations (> 20 mM) of the C_2 acids glyoxylate (2-oxoacetate) and glycolate (2-hydroxyacetate) also are required to stimulate the enzyme maximally (data not shown). Recently, others have observed maximal MnP activity with 500 μM oxalate (Kuan & Tien, 1993) although we observe optimal activity at ~2 mM oxalate (Figure 2). This suggests that oxalate is the physiologically relevant chelator (Barr et al., 1992; Kuan & Tien, 1993; Dutton et al., 1992). This led us in the present study to examine the effect of several organic acid chelators and pH on various steps in the manganese peroxidase catalytic cycle in order to better understand the unique effects of oxalate.

While optimal activity is observed at higher concentrations (>10 mM) of malonate and lactate, oxalate stimulates MnP optimally at low physiological concentrations (1–2 mM) (Table 1). In addition, at low concentrations of malonate and lactate, the reverse reaction (native enzyme oxidation to compound II) occurs. In contrast, this reverse reaction is not observed at any concentration of oxalate. Since the C_2 acids glyoxalate and glycolate exhibit behavior similar to malonate, our results suggest that oxalate may act in a unique manner. It is possible that the binding of C_3 chelators is sterically hindered. Furthermore, the C_2 chelators glyoxalate and glycolate are less effective chelators than oxalate. Thus, only oxalate is able to facilitate effectively the chelation and stabilization of Mn^{III}, resulting in optimal activity at relatively low oxalate concentrations. At high oxalate concentrations, a higher degree of Mn^{II} complexation occurs (Demmer et al., 1980; Taube, 1948), suggesting that dichelated Mn^{II} is not a good substrate. At the physiological concentration of oxalate, Mn^{III} forms a dioxalate complex, whereas Mn^{II} is monochelated (Demmer et al., 1980; Taube, 1948).

A monooxalate complex of Mn^{II} (Kuan et al., 1993), rather than aquo-Mn^{II} as previously suggested (Wariishi et al., 1992), reacts with oxidized forms of MnP. However, our data suggest that Mn^{III} is released from the enzyme in its dichelated form. Thus, further chelation apparently occurs after oxidation of Mn^{II} to Mn^{III}. Our results on compound I formation rates indicate that chelators do not bind directly to the iron(III) of the native enzyme. This does not preclude a binding site close to the heme. Inhibition of Mn^{III} reactions with other oxidizable molecules by higher concentrations of oxalate is probably caused by a higher degree of chelation of Mn^{II}, making it less reactive.

With our present state of knowledge, a satisfactory mechanism for compound II reduction by Mn^{II} in the presence of oxalate is shown in eqs 6 and 7:

$$\text{compound II} + Mn^{II}\text{ (monochelated)} \xrightarrow{\text{slow}} \text{native MnP} + Mn^{III}\text{ (monochelated)} \quad (6)$$

$$Mn^{III}\text{ (monochelated)} + \text{chelator} \xrightarrow{\text{fast}} Mn^{III}\text{ (dichelated)} \quad (7)$$

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